EuPA OPEN PROTEOMICS 8 (2015) 116–127

ScienceDirect

journal homepage: http://www.elsevier.com/locate/euprot

Aspirin-mediated acetylation of haemoglobin increases in presence of high glucose concentration and decreases protein glycation

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ARTICLE INFO

Article history:
Available online 8 May 2015

Keywords:
Protein glycation
Aspirin-mediated acetylation
Red blood cells
Haemoglobin
Mass spectrometry
Label-free

ABSTRACT

Glycation represents the first stage in the development of diabetic complications. Aspirin was shown to prevent sugars reacting with proteins, but the exact mechanism of this interaction was not well defined. We performed a quantitative analysis to calculate the levels of acetylation and glycation of haemoglobin, among others red blood cell (RBC) proteins, using a label free approach. After glucose incubation, increases in the acetylation levels were seen for several haemoglobin subunits, while a parallel decrease of their glycation levels was observed after aspirin incubation. These results suggest that, a mutual influence between these two modifications, occur at protein level.

1. Introduction

As diabetes progresses, the number of disease-specific complications has a tendency to increase. Among other risk factors, this is mostly due to chronic hyperglycaemia which promotes an uncontrolled, non-enzymatic modification of proteins called glycation. This post-translational modification (PTM) occurs between electrophilic glucose and nucleophilic primary amino groups of proteins (typically the N-terminal and ε amines of lysine) to generate a stable covalently bound intermediate, the Amadori compound. The latter represents the central core for the subsequent irreversible reactions of the glycation process [1]. The reaction's kinetics are enhanced by high and prolonged exposure to glucose, which in turn leads to the chronic health problems commonly observed in diabetes, including neuropathy [2], nephropathy [3], retinopathy [4] and cardiovascular diseases [5,6]. These disorders frequently appear several years after the onset of the illness, therefore, timely glycaemic control is of utmost importance in order to minimize the deleterious effects of glucose [7]. Today, blood glucose is typically monitored using the glycated haemoglobin (HbA1c) test considered, the gold standard for disease definition and the monitoring of anti-diabetes treatment [8]. Nevertheless, increasing evidence has shown that glycated haemoglobin may be affected by different inter-individual

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http://dx.doi.org/10.1016/j.euprot.2015.04.003

factors, leading to a "glycation gap" between the HbA1c and the level of mean plasma glucose [9,10]. This discrepancy can result from any one of a range of physiological variables that leads to an underestimate of the HbA1c levels in diabetic patients. These include any disorder that alters the lifespan of erythrocytes (red blood cells, RBCs) such as renal failure, haemolytic anaemia, iron-deficiency anaemia or a blood transfusion. Moreover, haemoglobin variants and adducts, which result from PTMs of haemoglobin (carbamylated Hb and pre-HbA1c), may alter the outcome of the test, thus generating method-specific interferences [11]. It should be noted that haemoglobin is the most abundant protein in RBCs, and the variation of its glycation levels is mostly due to its continuous interaction with circulating glucose, which also, influences other RBC proteins. As a consequence, hyperglycaemia has deleterious effects on the lifespan of RBCs, altering deformability and oxidative stress, which in turn lead to the hypertension and vascular complications [12–16] typical of diabetes.

Over the last few decades, it has been shown that the severity of these disorders can be suppressed, or at least relieved, by aspirin. Aspirin is a cornerstone treatment for the prevention of diabetic complications such as cardiovascular diseases. Although aspirin mediates its antithrombotic effects by inhibiting the cyclooxygenase (COX 1) enzyme, it not only effects platelet function, but also RBCs. Aspirin decreases the platelet reactivity amplified by RBCs through the down-regulation of the P-selectin and integrin IIb/IIIa, which in turn reduces the pro-thrombotic phenotype of RBCs [17,18]. Moreover, the interaction between platelets and RBCs in blood vessels, during thrombus formation, is strongly influenced by any reduced deformability of RBC membranes. This rheological mechanism can be reduced by aspirin, which decreases the rate of RBC aggregation [19,20]. Furthermore, evidence suggests that aspirin induces RBCs' nitric oxide synthase, which is well known to be helpful in regulating of the vascular tone and immune response [21]. The efficiency of all these processes seems to be reduced in RBCs by an acetyl-hydrolase activity that rapidly decreases the half-life of aspirin in blood [22–24]. Despite the significant number of studies that have attempted to understand the separated roles of non-enzymatic glycation and aspirin acetylation on the biological processes of RBCs, the exact mutual and synchronous interaction between these two modifications has so far been poorly investigated.

In this study, we carried out the first analyses of the simultaneous impact of protein glycation and aspirin acetylation on RBC proteins, with a special emphasis on haemoglobin. An experimental mass spectrometry (MS) approach, previously applied to serum albumin [25] and human plasma [26], was used to obtain qualitative information, by detecting acetylation and glycation sites, as well as quantitative reports determining the levels of these modifications.

2. Materials and methods

2.1. Chemicals

EDTA (10.8 mg) – Vacutainer tubes were from BD Vacutainer®. Dulbecco's phosphate-buffered saline (DPBS; 1X, pH 7.4) was from Invitrogen™. NaCl 0.9% was from Bichsel. Mouse anti-human CD235a, Glycophorin A/RPE antibody was from Dako. Aspirin was from Aspegic Inject®. EDTA-free protease inhibitor cocktail (PIC) tablets were from Roche. Anti-human Nε-acetyl-lysine monoclonal antibody was from Cell Signalling Technologies®. ECL™ detection reagent was from GE Healthcare. Protein assay dye reagent concentrate (liquid) was from Bio-Rad. D-glucose [$^{12}C_6$] (lyophilized powder, ≥99.5%), Naphtol Blue Black (lyophilized powder, dye content *ca* 80%), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP; 0.5 M, pH 7.0), iodoacetamide (IAA, crystalline, ≥99%), endoproteinase Glu-C from *Staphylococcus aureus* V8 (lyophilized powder, 500 U), HPLC-grade water (CHROMASOLV®) and HPLC grade acetonitrile (ACN; CHROMASOLV®, ≥99.9%) were purchased from Sigma–Aldrich®. Bovine serum albumin (BSA, lyophilized powder, ≥96%), triethylammonium hydrogen carbonate buffer (TEAB; 1 M, pH 8.5), ammonium acetate (NH_4Ac, solid, 98.0%), magnesium chloride ($MgCl_2$, solid, ≥99.0%), acetic acid (99.5%), and formic acid (FA; 98.0%) were from Fluka.

2.2. RBC preparation and lysis

RBC fraction was obtained by centrifuging whole blood from three blood bank donors. Briefly, EDTA-Vacutainer tubes containing blood were centrifuged at 900 rpm, at 37 °C, to remove the platelet rich plasma fraction. Then, the remaining part was centrifuged at 3500 rpm for 10 min to separate and remove the plasma from the RBCs. Carefully 1 mL of erythrocytes was aspirated from the bottom of the tube and transferred to a Falcon tube. RBCs were washed four times with 0.9% NaCl and centrifuged at 3690 rpm for 10 min to minimize the presence of contaminants. Before the final washing step, 100 μL of cell suspension was used to determine the concentration RBCs using a Sysmex KX21 N haematology analyser (Sysmex Corporation). A sample concentration of 2.51×10^6 cells/μL was diluted down to 10^4 cells/μL and incubated with an anti-human antibody against CD235a (Glycophorin A) for subsequent fluorescence-activated cell sorting (FACS) analysis. The level of cell purity was assessed using an Accuri C6 fluorescence flow cytometer (BD Accuri™). After sedimentation, the supernatant was removed, and a cell pellet (~1 mL) was lysed by adding eight volumes of deionized water. Cell debris was separated by centrifugation at 13,000 rpm for 30 min at 4 °C, and protease inhibitors were added to the RBC extract at a dilution of 1:7 before aliquoting. The three RBC protein extracts were then pooled together.

2.3. RBCs incubation with aspirin and glucose

Four aliquots of 225 μL of RBC protein extract were diluted in phosphate buffer to reach a final volume of 400 μL, and were subsequently incubated with (1) 500 μM aspirin for 30 min at 37 °C; (2) 30 mM glucose for 24 h at 37 °C; and (3) sequentially, with 30 mM glucose at 37 °C for 24 h followed by 500 μM aspirin for 30 min at 37 °C. As negative control, a further aliquot was incubated using the same time periods, but without glucose and aspirin. Additional five conditions were created in which 100 μL of RBC extract were incubated for 24 h with increasing glucose concentrations (0, 10, 30, 50 and 100 mM) followed by 30 min of aspirin exposure at 37 °C. As a positive control,

20 mM aspirin were incubated in the absence of glucose. After incubation, protein amounts were determined using the Bradford assay, and BSA was used to create a standard calibration curve.

2.4. Western blot analysis of aspirin-mediated acetylation

Samples of 5 μg were separated using electrophoresis on a 15%T/2.67%C polyacrylamide gel, and protein transfer took place for 1 h at 350 mA. Membranes were stained with Amido black to highlight the protein bands [27], and, then, were washed with water to minimize background. A western blot test was performed using an anti-human antibody against N ε-acetyl-lysine (1:5000). ECL™ reagents were used to detect the acetylation signal. The intensity of the immunoreaction (detected by ECL) was normalized over the total protein signal of the corresponding band stained with Amido black. Each sample was assessed using three experimental replicates ($N = 3$).

2.5. Protein purification and Glu-C digestion

After incubation, glucose, aspirin and salts were removed from the samples using Amicon Ultra-0.5 mL, Ultracel® 3 K membrane (Millipore™) devices, and purified proteins were then reconstituted in 500 mM TEAB, pH 8.5. The amount of protein was estimated using the Bradford assay, with BSA to perform the calibration curve. Samples were separated into two groups in order to analyze the acetylated and glycated peptides separately for each experimental set. An amount of 200 μg and 1 mg of protein was dissolved in 400 μL of TEAB for the acetylation and glycation study, respectively. Then, cysteine residues were reduced with 5 mM TCEP (4 μL) by incubation of the reaction mixture for 1 h at 60 °C. Free sulfhydryl groups were alkylated with 20 mM IAA (10 μL) for 30 min at room temperature in dark conditions. Freshly prepared endoproteinase Glu-C (1 mg/mL) was added in a ratio of 1:10 (w/w), and digestion took place for 16 h at 37 °C. For acetylation analysis, digestion mixtures were lyophilized under speed vacuum and reconstituted in 5% ACN/0.1% FA for the following desalting step. For the glycation analysis, peptide digests were evaporated and reconstituted in mobile phase A (200 mM NH_4Ac, 50 mM $MgCl_2$, pH 8.1) to obtain an estimated concentration of 16 mg/mL, for the enrichment of glycated peptides.

2.6. Enrichment of glycated peptides using boronate affinity chromatography

Reconstituted peptides were fractionated by boronate affinity chromatography by the interaction between the hydroxyl-groups of glycated peptides, present at low concentrations, and boronic acid, as previously described [28,29]. For this purpose, samples (50 μL) were injected in a Waters 600E HPLC system equipped with a TSK-Gel boronate affinity column from Tosoh Bioscience (7.5 cm length × 7.5 mm inner diameter, i.d., 10 μm particle size). An isocratic chromatographic method based on three steps was used to separate non-glycated from glycated fractions. Step 1 was a 0–10 min, 100% mobile phase A (200 mM ammonium acetate, 50 mM magnesium chloride, pH 8.1) for the retention, under alkaline conditions, of glycated peptides by esterification of the 1,2 cis-diol group of glucose moieties and the hydroxyl groups of boronate ligands with elution of non-glycated peptides. In step 2, 10–20 min, glycated peptides are eluted with 100% mobile phase B (100 mM acetic acid) under acidic conditions; and step 3, 20–30 min with 100% mobile phase A for the equilibration of the column to the initial conditions. The glycated fraction was collected for subsequent evaporation and reconstitution in 5% ACN/0.1% FA. Then, peptides were desalted and pre-concentrated prior to LC–MS/MS analysis. This was carried out with C_{18} MacroSpin Column™ (Harvard Apparatus), and peptides were eluted in 400 μL of 50% ACN/0.1% FA and then evaporated to dryness before reconstitution in 5% ACN/0.1% FA.

2.7. LC–MS/MS detection of acetylated and glycated peptides

Peptide digests were analyzed by electrospray ionization in positive ion mode (1.9 kV ionization voltage), on an Orbitrap hybrid linear ion trap (Thermo Fisher). Nanoflow was performed using a Waters NanoAquity HPLC system consisting of a pre-column (100 μm inner diameter and 18 mm in length) packed with C_{18} resin, where peptides were initially trapped at a flow rate of 3 μL/min in water/ACN (95/5 v/v) with 0.1% FA. Peptide separation was then performed using an ACN gradient developed in an analytical column packed with C_{18} resin, at the flow rate of 200 nL/min with mobile phase A (water, 0.1% FA) and B (ACN, 0.1% FA). A data-dependent tandem mass spectrometry method was used for analysis of acetylated and glycated proteins with a precursor-ion scan range of 400–2000 m/z and a resolving power of 60,000. Fragmentation of the five most abundant ions, detected from the MS1 survey scan, was performed in the octopole collision cell at the rear of the C-trap (normalized collision energy, 35%), followed by Orbitrap detection with a resolving power of 7500 and a dynamic exclusion of 120 s, to minimize repeated analysis of the same precursor ion. The fragment ion isolation window was set to 2.5 m/z units. Precursor ions of charge state 1+ were excluded for the data-dependent acquisition. All analyses were carried out using three replicate injections.

2.8. Data analysis

After data-dependent acquisition, peak lists were generated from the raw data using EasyProtConv v1.5 software, and the resulting data files were searched for matches against the UniProtKB/Swiss-Prot database (Release June 13, 2012, 659,907 entries) using the EasyProt v2.3 (build 751) tool [30]. Because this study analyzed human RBCs, we specified *Homo sapiens* taxonomy for database searching. The acquired high resolution data allowed the scoring model to use an accuracy of 6 ppm for fragment ions and 10 ppm for their precursor ions. Common amino acid modifications detected were carbamidomethylation of cysteine residues (57.0215 Da) and oxidized methionine (15.9949 Da), which were set as fixed and variable modifications, respectively. For the analysis of aspirin and glucose modifications, acetylation of lysine residues or N-terminal positions (42.0100 Da), was selected as variable modification and glycation of lysine and arginine residues

or N-terminal positions (162.0523 Da) was selected as variable modification, as well. The number of modifications per peptide was set to a maximum of 2 in order to reduce the search space of all possible combinations of variable modifications. Endoproteinase Glu-C was selected as the cleavage enzyme, with three potential missed cleavages allowed. The minimum peptide length was 8 amino acid residues, with a minimum peptide z-score of 4. Three analytical injections per sample were each analyzed independently, providing replicate data values. The peak list files obtained for each technical replicate were merged and used to search the database. For the significance of peptide identification, we used a false-discovery rate of 1%. Calculation of the peptide false discovery rate (FDR) is automatically performed by EasyProt v2.3 as $\%FDR = M_R/M_F \times 100$, where M_R and M_F are the number of PSMs (Peptide-Spectrum Matches) from the reverse and the forward database searches respectively.

2.9. *Peptide quantification*

Quantitation of acetylated and glycated peptides from RBC samples was carried out using Nonlinear Dynamics' Progenesis software. Briefly, after the accurate alignment of *m*/*z* and Rt (retention time) features for every chromatographic run in each testing group, a global peak list file, containing all the features detected, was created and used to query the database, and from the identification results, a pepXML file was exported to Progenesis. Each identified peptide was then associated to its relative feature and its raw abundance was normalized multiplying it by a global scaling factor. This scaling factor is defined by the anti-log of the average of the log ratios between the run being normalized and the reference run.

3. Results

3.1. *Identification of acetylated and glycated proteins in RBCs*

This study aimed to evaluate the mutual interaction between protein glycation and aspirin-induced acetylation on RBC cytosolic proteins. After isolating RBCs from whole blood, the purity of the cell population was assessed using FACS through cell-line specific antibodies conjugated with fluorochromes at different emission wavelengths. This analysis revealed that a highly homogeneous RBC distribution had been obtained, with a very low degree of platelet and leucocyte contamination, as shown in Supplemental Fig. 1A and B. Next, after lysis of RBCs and protein extraction through centrifugation, 30 mM glucose incubation was selected to mimic a gluco-toxicity level; 500 μM aspirin concentration was used as this is within the usual therapeutic blood ranges. Aspirin's half-life *in vivo* is approximately 20 min; in order to be more confident with its kinetics in this analysis, an incubation period of 30 min was chosen [31].

A total number of 96 proteins, including acetylated, glycated and unmodified ones, were identified in this study in the cytosolic fraction of RBCs. The name of the proteins with associated their accession number, the sequence coverage and the number of detected peptides with which protein has been identified, are listed in Supplemental Table 1. The MS acetylation pattern of RBCs was characterized using the four test conditions. Table 1 shows the list of acetylated proteins that were identified with information about the peptides detected for each protein with the acetyl attachment sites. A more detailed list of acetylated proteins, their respective acetylated peptides and modification sites, with information about the experimental masses, the *m*/*z* and Rt values and the scan name is furnished in Supplemental Table 2. Among them, haemoglobin subunits α, β and δ show the most of assigned acetylation sites. Other RBC proteins, including peroxiredoxin 2, superoxide dismutase and carbonic anhydrase 1, were found to be significantly acetylated by aspirin. The percentage of acetylated proteins reached in this study is 15.62%. The MS glycation profile of RBC proteins was obtained after their enrichment with BAC. The proportion of non-glycated peptides identified in the retained fraction was below 35% across all the test conditions. As shown in Table 2, most of the glycated sites identified belong to haemoglobin α, β and δ subunits. Other proteins were also distinguishable in the presence of high glucose concentrations such as peroxiredoxin 1 and 2 and carbonic anhydrase 1. A more detailed list of glycated proteins with their glycated peptides and the annotation of their modification sites are provided in Supplemental Table 3, with information about the *m*/*z* and Rt value for each modified peptide and the respective scan name. The percentage of glycation before and after enrichment was estimated at 1.72% and 21.87%, respectively.

To discover which proteins underwent both acetylation and glycation modifications, the data sets were compared. A total of six specific RBC proteins were found to be target of both modifications (α, β and δ subunits of haemoglobin, peroxiredoxin 2, superoxide dismutase and carbonic anhydrase 1. The six proteins had 10 sites which were found to be both acetylated and glycated. Five sites belonged to haemoglobin β in the N-terminal Val and in Lys residues located at positions 9, 18, 59 and 96. All these sites had previously been found to be the targets of glycation [32] and aspirin-induced acetylation [33,34]. The other five acetylated/glycated sites were identified as residues Lys 11 and Lys 16 of haemoglobin subunit α, Lys 95 of haemoglobin subunit δ, Lys 183 of peroxiredoxin 2 and Lys 45 of carbonic anhydrase 1.

To assess the presence of a specific amino acid motif in proximity of these 10 sites, we carried out a motif analysis using the primary structure of the corresponding peptide sequences. The sequences were loaded on WebLogo software [35], specifying 20 amino acids prior to and after the modification site. The frequency of occurrence of each amino acid at a given position is graphically represented by its size. Looking at the logo (Fig. 1A), acidic side chain residues (aspartic acid and glutamic acid) and basic side chain residues (lysine) were identified to be highly representative in close proximity of the modification site. In addition, hydrophobic short chain amino acids like alanine, valine and leucine, were observed to occur most frequently in acetylated/glycated peptides identified in this study. Furthermore, the study of sequence motifs was integrated with a tertiary (3D) structure analysis in order to figure out the importance of specific amino acids located in the neighbouring environment of the potential reactive site. Using PyMOL v1.5 program, we observed that several reactive

Table 1 – Acetylated proteins with information about the detected peptides and the position of the acetylation sites identified in RBCs, after aspirin incubation, after glucose incubation and after glucose and aspirin incubation.

Protein	Peptide	Position	RBCs	RBCs + Aspirin	RBCs + Glucose	RBCs + Glucose + Aspirin
Haemoglobin subunit beta	VHLTPEEKSAVTALWGKVNVDE	1–22	V1, K17	V1, K8	V1	V1, K17
	VHLTPEEKSAVTALWGKVNVDEVGGE	1–26	x	V1, K17	x	K8, K17
	VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFE	1–43	K8, K17	K8, K17	K8, K17	K8, K17
	SFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSE	44–90	x	K59, K61	K59	K59, K61
	LHCDKLHVDPE	92–102	x	K96	x	K96
Haemoglobin subunit delta	NFRLLGNVLVCVLARNFGKEFTPQMQAAYQKVVAGVANALAHKYH	102–146	K120	K120	K120	K120
	LHCDKLHVDPE	91–101	x	K95	x	K95
Peroxiredoxin-2	HGEVCPAGWKPGSDTIKPNVDDSKE	167–191	x	K183, K190	x	K183, K190
	VCPAGWKPGSDTIKPNVDDSKE	170–191	x	K183	x	K183, K190
Haemoglobin subunit alpha	VLSPADKTNVKAAWGKVGAHAGE	1–23	x	K11	x	K11, K16
	VLSPADKTNVKAAWGKVGAHAGEYGAEALE	1–30	x	K16	x	K11, K16
Carbonic anhydrase 1	TKHDTSLKPISVSYNPATAKE	38–58	x	K45	x	K45
Superoxide dismutase [Cu-Zn]	ATKAVCVLKGDGPVQGIINFE	1–21	A1	A1	A1	A1
	ATKAVCVLKGDGPVQGIINFEQKE	1–24	A1	A1	A1	A1
Catalase	ADSRDPASDQMQHWKE	1–16	A1	A1	A1	A1
Glutathione S-transferase omega-1	SGESARSLGKGSAPPGPVPE	2–21	S2	S2	S2	S2
Low molecular weight phosphotyrosine protein phosphatase	AEQATKSVLFVCLGNICRSPIAE	2–24	A2	A2	A2	A2
RNA-binding protein 12	KIDMIRKRLQNFSYDQREMILNPE	513–536	K519	x	x	x
Flavin reductase (NADPH)	AVKKIAIFGATGQTGLTTLAQAVQAGYE	1–28	x	K3	K3	A1, K3
SH3 domain-binding glutamic acid-rich-like protein 3 (SH3BP-1)	SGLRVYSTSVTGSREIKSQQSE	2–23	x	x	S2	S2
AT-rich interactive domain-containing protein 3A	GPGEEHFEDMASDEDMKPKWEEEE	108–131	x	x	x	K124
Alpha-haemoglobin-stabilizing protein	ALLKANKDLISAGLKE	2–17	x	A2	x	x
Peptidyl-prolyl cis-trans isomerase A, N-terminally processed	AMERFGSRNGKTSKKITIADCGQLE	140–164	x	R143	x	R143

Table 2 – Glycated proteins with information about the detected peptides and the position of the glycation sites identified in RBCs, after aspirin incubation, after glucose incubation and after glucose and aspirin incubation.

Protein	Peptide	Position	RBCs	RBCs + Aspirin	RBCs + Glucose	RBCs + Glucose + Aspirin
	VHLTPEEKSAVTALWGKVNVDE	1–22	V1, K8	K8	V1, K8, K17	V1, K8, K17
	VHLTPEEKSAVTALWGKVNVDEVGGE	1–26	K8	K8	K8, K17	K8, K17
	KSAVTALWGKVNVDE	9–23	x	x	x	K9
Haemoglobin subunit beta	VGGEALGRLLVVYPWTQRFFE	24–44	x	R31	R31, R41	R31, R41
	SFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSE	45–91	x	x	K59, K65	x
	LHCDKLHVDPE	92–102	x	x	K96	K96
	FTPPVQAAYQKVVAGVANALAHKYH	123–147	x	x	K133	K133
	LHCDKLHVDPE	91–101	x	x	K95	K95
Haemoglobin subunit delta	VHLTPEEKTAVNALWGKVNVDAVGGE	1–26	x	x	x	K8
	VHLTPEEKTAVNALWGKVNVDAVGGEALGRLLVVYPWTQRFFE	1–43	x	x	K8, K17, R30	K8
	GGLGPLNIPLLADVTRRLSEDYGVLKTDE	93–121	x	x	K118	x
Peroxiredoxin-2	HGEVCPAGWKPGSDTIKPNVDDSKE	167–191	x	x	K176, K183	K176, K183
	VCPAGWKPGSDTIKPNVDDSKE	170–191	x	x	K176	K176
	VLSPADKTNVKAAWGKVGAHAGE	1–23	K7, K11	K7	K7, K11	K7, K11
Haemoglobin subunit alpha	VLSPADKTNVKAAWGKVGAHAGEYGAE	1–27	K7, K11	K7	K11	K11
	VLSPADKTNVKAAWGKVGAHAGEYGAEALE	1–30	x	x	K11, K16	x
Carbonic anhydrase 1	TKHDTSLKPISVSYNPATAKE	38–58	K57	K57	K45, K57	K39, K45, K57
Superoxide dismutase [Cu-Zn]	ATKAVCVLKGDGPVQGIINFE	1–21	x	x	K9	K9
	ATKAVCVLKGDGPVQGIINFEQKE	1–24	x	x	K9	K9
	CFLQHKDDNPNLPRLVRPE	101–119	K106	K106	x	x
	KCCAAADPHECYAKVFDE	359–376	K372	K372	x	x
Serum albumin	LRDEGKASSAKQRLKCASLQKFGE	185–208	K195	K195, R197	x	R197
	RAFKAWAVARLSQRFPKAE	209–227	R218	R218	x	x
	VSKLVTDLTKVHTE	231–244	K233	K233	K233	K233
Peroxiredoxin-1	VCPAGWKPGSDTIKPDVQKSKE	171–192	x	x	K177, K184	K177
Putative IQ and AAA domain-containing protein 1-like	GPDMKEKMKE	289–298	K293, K297	x	x	x
Obscurin-like protein 1	LSREDAPVRWYKDGLE	1013–1028	R1015	x	x	x
WW domain-binding protein 4	KASKEFAAMEAAALKAYQE	60–78	x	K63	x	x
TATA element modulatory factor	KTRSIMAEELVKLTNQNDELEE	1012–1033	x	R1014, K1023	x	x
Protein kinase C and casein kinase substrate in neurons protein 1	KRLVFLKE	240–247	x	R241	x	K246
Nck-associated protein 5	TVNIMVYQEKLSEEERKHKEALE	111–133	x	x	K120, R126	x
Kinesin-like protein KIF20A	TLHVAKFSAIASQLVHAPPMQLGFPSLHSFIKEHSLQVSPSLE	493–535	x	x	K498	x
R3H domain-containing protein 2	KSTKDVSEKEDKDKNKE	115–131	x	x	K118, K128	x
Protein S100-Z	PTQLEMAMDTMIRIFHRYSGKE	1–22	x	x	R13, R17	x
Cyclin-dependent kinase 11B	KRKEKRKHARVKE	70–82	x	x	R71	x
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	TKTPNLRMSE	16–25	x	x	R22	x
Golgin subfamily B member 1	QAAQQVVREKDARFETQVRLHE	213–234	x	x	x	K222
Ninein (hNinein) (GSK3B-interacting protein)	RIAALKNE	429–436	x	R429	R429	R429

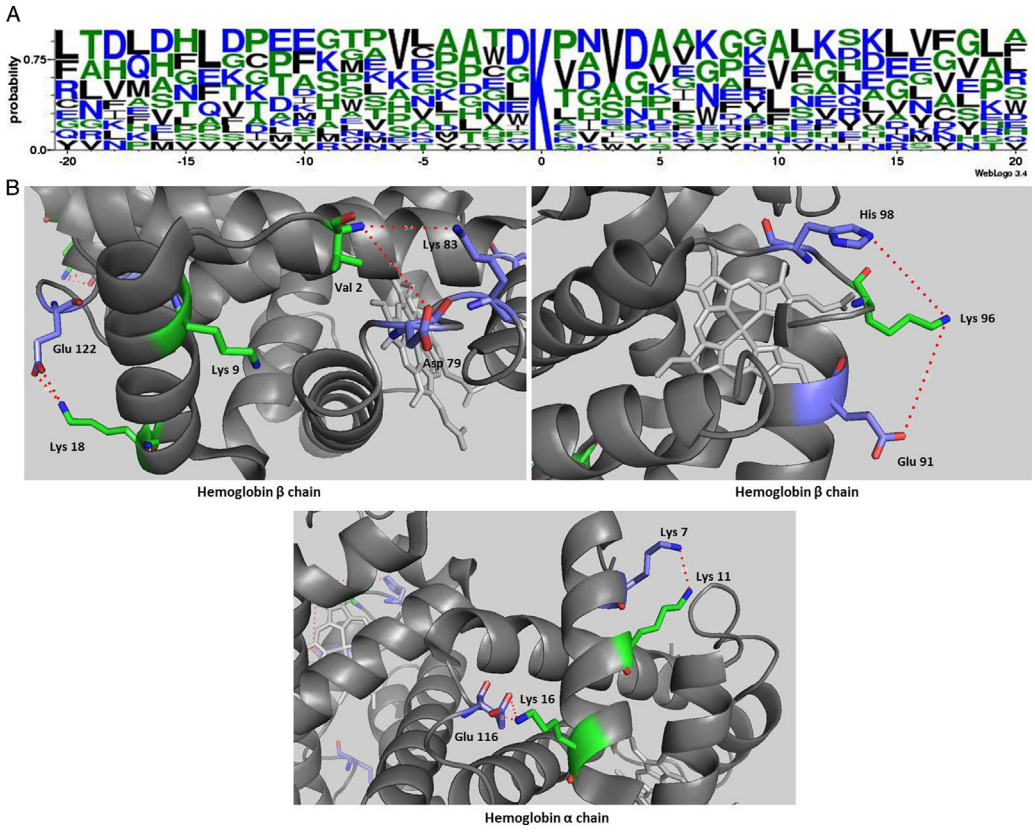

Fig. 1 – Motif logo analysis of acetylated and glycated peptides detected in RBCs after glucose and aspirin incubation. The X-axis represents the neighbouring residues (−20 to +20) around the modified lysine (0 position) in the primary sequence structure; the Y-axis represents the frequency of occurrence of each amino acid present at each location (A). Protein tertiary structure analysis shows the spatial proximity around the modification sites of haemoglobin β and α chains that are detected as both acetylated and glycated. Red dashed lines represent the distances between the modified residues (green) and the neighbouring amino acids (blue) (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sites found in this study to be both acetylated and glycated in haemoglobin α and β chain, were located in the spatial proximity of acidic and basic amino acids (Fig. 1B), confirming, once again, the relevance of this class of residues for lysine modification by aspirin and glucose. These results are in line with those obtained by Zhang et al. [36] on RBCs from diabetic patients, highlighting the specificity of site modification.

3.2. *Assessment of the levels of acetylation and glycation of carbonic anhydrase 1*

The label free approach described in the Experimental Section was used to quantify the extent of aspirin-acetylation and non-enzymatic glycation on RBC proteins. Supplemental Tables 4 and 5 show the lists of acetylated and glycated proteins quantified, with information of the peptides detected for each protein, the site of modification and the relative abundance across all test conditions, respectively.

Most of the proteins quantified are specific to RBCs, such as haemoglobin subunit β (4 acetylated peptides, 6 glycated peptides), haemoglobin subunit α (2 acetylated peptides, 3 glycated peptides), haemoglobin subunit δ (2 acetylated peptides, 2 glycated peptide), peroxiredoxin 2 (2 acetylated peptides, 3 glycated peptides) and carbonic anhydrase 1(1 acetylated peptide, 1 glycated peptide), to cite but a few. Quantification measurements from acetylated and glycated RBC proteins were initially used in a principal component analysis to highlight the main differences between the four groups and to represent the inter- and intra-group variability across the replicates. These results showed that the four sample groups were separate (with a slight degree of overlap between them) for both the acetylation (Supplemental Fig. 2A) and glycation data set (Supplemental Fig. 2B), thus providing a first general proof of the mutual effects of aspirin and glucose on RBC proteins.

This behaviour strongly reflects the response of aspirin acetylation and glycation at peptide/site level, as shown for peptide TKHDTSLKPISVSYNPATAKE from carbonic anhydrase 1 (Fig. 2). In particular, a significant increase in its aspirin-mediated acetylation level is evident with prior glucose incubation (Fig. 2A), whereas, a significant decrease of protein glycation is observed in the presence of glucose after aspirin exposure, in comparison to glucose incubation alone (Fig. 2B). The fragment spectra of this peptide (the most representative peptide identified in this study) confirm the presence of acetylation (Fig. 2C) and glycation (Fig. 2D) on

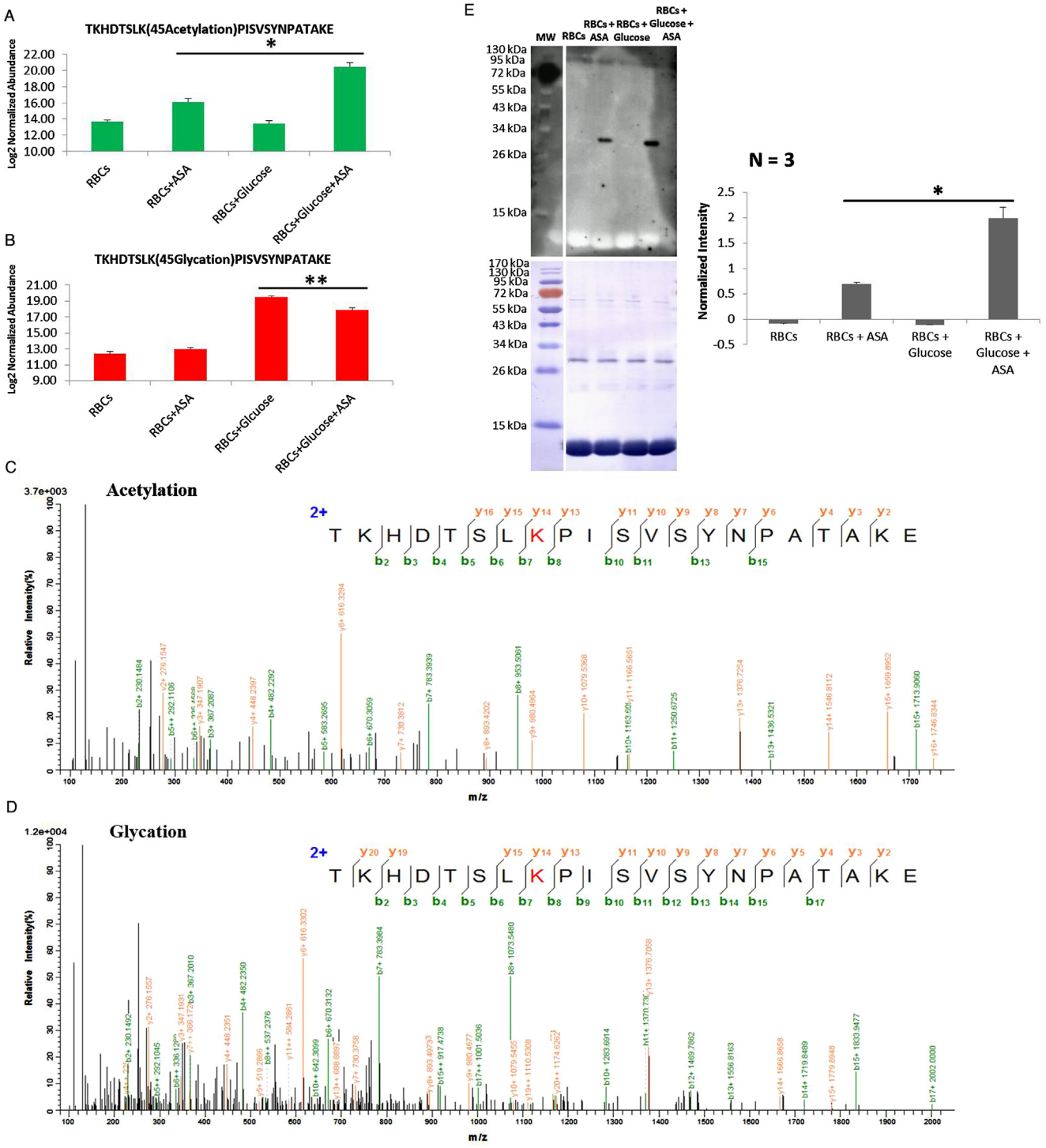

Fig. 2 – Quantification of the peak abundances of carbonic anhydrase peptide TKHDTSLKPISVSYNPATAKE in its acetylated (green bars) (A) and glycated (red bars) state (B). Annotated spectra of the same peptide in its acetylated (top spectrum) (C) and glycated (bottom spectrum) states (D). Acetylation level measured by western blot shows a significant increase after 30 mM glucose incubation followed by 500 μM aspirin, compared to aspirin incubation alone, for the 28 kDa protein band detected (carbonic anhydrase 1) (E). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

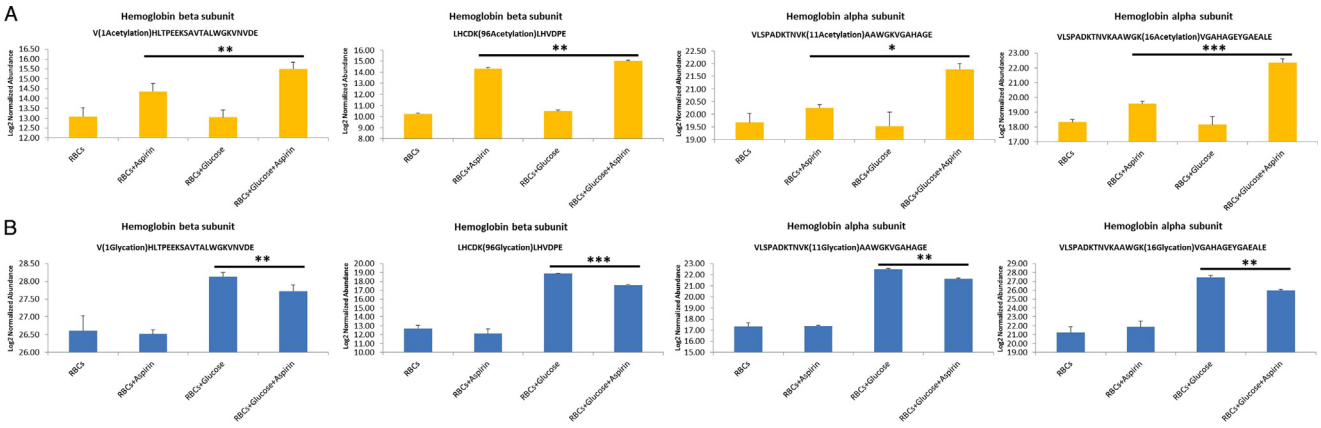

Fig. 3 – Quantification of the peak abundances of 4 acetylated (orange bars) and glycated (blue bars) sites from four representative peptides belonging to haemoglobin β and α subunit, in all the test conditions (native, 500 μM aspirin, 30 mM glucose, 30 mM glucose + 500 μM aspirin). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lysine 45. These results were clearly confirmed by western blot tests using an anti-acetylation antibody and the same sample conditions ($N = 3$). Indeed, a significant difference in the acetylation reactions for the protein band at 28 kDa, was seen when comparing the different lanes (Fig. 2E). The acetylation signal in RBCs incubated with glucose followed by aspirin (Fig. 2E, lane 4) was significantly higher than the signal after aspirin incubation alone (Fig. 2E, lane 2), and no signal was detected in either native RBCs or after glucose incubation alone (Fig. 2E, lanes 1 and 3). Glu-C in-blot digestion of the band slice followed by LC–MS/MS identification, allows the unambiguous assignment of the carbonic anhydrase 1 (data not shown).

Interestingly, when comparing RBC protein extracts incubated with increasing glucose concentrations followed by 500 μM aspirin exposition, a significant increase in the acetylation levels was evident at 30 mM glucose, in comparison to 10 mM or no glucose (Supplemental Fig. 3, lanes 1–3), but no further significant gain was observed at 50 and 100 mM glucose (Supplemental Fig. 3, lanes 4–5).

3.3. *Quantitation of acetylation and glycation sites on Haemoglobin*

The same trend in the variation of the acetylation and glycation levels that was shown for carbonic anhydrase 1, was also observed for other RBC proteins, including the haemoglobin α and β subunits. The quantitation of the seven Hb sites found to be common targets of both PTMs, revealed a significant variation after the sequential incubation of glucose and aspirin. Fig. 3A shows the level of acetylation of two peptides, from haemoglobin β and α subunits, respectively. Again, a significant increase in aspirin acetylation, after glucose incubation, was evident for Val 1 and Lys 96 from the β subunit and for Lys 11 and Lys 16 from the α subunit, compared to aspirin incubation alone. Furthermore, a parallel decrease of glycation levels was observed after 30 min aspirin exposure in the presence of glucose, compared to glucose incubation alone for the four representative sites (Fig. 3B). Of these, the Val residue at the N-terminal position of the haemoglobin β chain is of interest because it is the target site for measuring the %HbA1c. Supplemental Fig. 4A shows the variation of the peak area of the haemoglobin β subunit N-terminal peptide VHLTPEEKSAVTALWGKVNVDE containing acetylated valine, across all test conditions. The abundance of the acetylated peptide shows a 1.4-fold increase after glucose and aspirin incubation compared to aspirin incubation alone. The corresponding peptide fragmentation spectrum confirms the presence of acetylation on the N-terminal valine residue, as shown in Supplemental Fig. 4B.

In contrast, Supplemental Fig. 5A represents the differences in the peak abundances of the same peptide with the glycated N-terminal valine; its levels show a 1.5-fold decrease when aspirin is incubated in the presence of glucose compared to glucose incubation alone. MS/MS analysis allowed the precise assignment of the glycated residue on the peptide sequence (Supplemental Fig. 5B). Similar behaviour was also observed for the other haemoglobin sites, reinforcing the assumption that high glucose concentrations might favour aspirin acetylation, which in turn protects proteins from further glycation.

4. Discussion

Human blood is usually the first microenvironment in which drugs and metabolites interact with the body's cellular components and for this reason it is the most commonly used bio-fluid for biomarker measurements. HbA1c is a typical example, representing a cornerstone biomarker in the diagnosis and monitoring of diabetes mellitus. Haemoglobin is the most abundant protein in RBCs (~97%), and the level of its glycated state (%HbA1c) should generally correlate with the status of blood glucose for long-term glycaemic control [37]. However, divergences between HbA1c levels and clinical measures of glycaemia (*e.g.*, fructosamine assay), have led to the theory that this "glycation gap" leads to a slight but critical misinterpretation of the real glycaemic state [10,38]. To date, the mechanisms underlying such discordance seem

attributable to a great variety of factors, such as genetic predisposition, age or race and the administration of certain drugs [39–41]. Furthermore, haemoglobin variants, high levels of haemoglobin F and derivatives represent the most common analytical interferences influencing the interpretation of HbA1c results [11,42].

It has previously been shown how aspirin affects the biological functions of RBCs, decreasing their tendency to aggregate and to stimulate the recruitment of platelets and leucocytes [17]. However, the molecular process through which the effects of aspirin and glucose influence each other in RBCs at the protein level, was little known. Using fluorescence and circular dichroism experiments, Bakhti et al. showed that glycation induces conformational changes in the secondary structure of haemoglobin, which are in turn prevented by aspirin [43]. We recently examined the impact of aspirin on human plasma in the presence of a high glucose concentration, theorizing that the observed interplay between these two PTMs may be the result of conformational changes in proteins [26]. The present study used a combination of high resolution tandem mass spectrometry coupled with label-free quantitation in order to measure the extent of acetylation and glycation on RBC proteins after incubation with a high glucose concentration (30 mM) followed by exposure to a therapeutic dose of aspirin (500 μM). The high accuracy offered by the HCD-MS/MS mode for fragment ions, coupled with Orbitrap detection [44], allowed us to identify acetylated and glycated peptides in each test condition. Six specific RBC proteins were shown to be preferentially acetylated and glycated, and on them, 10 sites were characterized as the targets for both PTMs. Most of these are associated to haemoglobin chains, probably due to the high level of this protein in RBC cytosol. By using the WebLogo interface [35], a motif analysis of the primary structures in proximity of these 10 residues revealed a high number of acidic (Asp and Glu) and basic amino acids (Lys), that may favour lysine modification, as previously suggested [33,45,46]. However, information on the sequence primary structure does not reflect the spatial environment of the potential reactive sites, and therefore an evaluation of which amino acids were close to the reactive lysine was carried out on the 3D structure of proteins, using PyMOL v1.5 software. In this way, it was possible to determine that, despite the fact that all the sites were located on the surface of the protein, most of them were also in close proximity to an acidic or basic amino acid (from 2.7 to 7.7 Å) in the tertiary structure of the protein. This suggested the importance of these classes of residues in the promotion of the modifications caused by aspirin and glucose.

Quantitative data demonstrated a significant increase of the degree of acetylation when aspirin was incubated in the presence of glucose, compared to aspirin incubation alone, for the majority of the acetylated sites detected. Among them, the Val 2 residue of the haemoglobin β subunit is of importance because of its role, in its glycated state, in monitoring the HbA1c level for glycaemic control, as previously mentioned.

These observations were also confirmed using a western blot test in which the state of acetylation of carbonic anhydrase increased significantly when glucose and aspirin are incubated sequentially. Moreover, the increase in the degree of acetylation seemed to be dependent on the level of glucose, with a critical threshold at 30 mM. This could support the interpretation by which protein glycation favours acetylation in a process governed by a variation of intramolecular chemo-selectivity, potentially due to conformational changes that alter a dynamic equilibrium between non-reactive and reactive sites towards both PTMs. A similar trend was previously described for different plasma proteins showing the same effect of glucose on aspirin-acetylation [26]. Plasma and RBCs are the most representative components of whole blood, 58% and 42% respectively (with a very low percentage of leukocytes and platelets) and, consequently, their proteins interact more likely with glucose compared to the other two blood compartments. In line with our findings, conformational changes exerted by glycation could in turn make those proteins more prone to be influenced by aspirin. This could explain the match observed in the acetylation response to glucose for both plasma and RBCs data set.

With regard to glycation, a significant decrease in its level was observed after 30 min of exposure to aspirin, compared to glucose incubation alone, for most of the glycated sites. This again highlights the role exerted by aspirin in reducing further glycation. One noteworthy aspect of these results derives from the influence that aspirin exerts on the Val 2 residue of haemoglobin β chain. Indeed, the glycated form of this N-terminal amino acid is commonly used in clinical measurements of HbA1c levels, which correlate with mean blood glucose levels for the diagnosis and follow-up of diabetic complications and treatment. However, the HbA1c test can be biased by the presence of haemoglobin variants (S, C, D, E and F) which can affect the measurement and make it unreliable. Although these variants do not cause haemolytic disease in their heterozygous form, and although their glycation level is the same as that for HbA1c, they can interfere with measurements of HbA1c depending on which test method is used [47]. The presence of different point mutations (HbE, HbD) or the replacement of β chains with γ or δ chains (β-thalassemia), alters the physicochemical properties of haemoglobin (pI and the extent of glycation). This favours the detection and quantitation of haemoglobin variants using methods based on ion exchange chromatography or capillary electrophoresis but not the use of methods based on boronate affinity chromatography and immunoassay [42]. Besides the impact of haemoglobin variants, the present study showed that aspirin reduces the glycation level of Val 2 by up to 30%; thus, *in vitro* diagnostic testing may routinely underestimate %HbA1c. This is relevant and important in the diagnosis and management of diabetes mellitus: aspirin is the most widely used drug to reduce the risk of cardiovascular disease in diabetic patients, but might in fact be a variable that leads to differences between observed and predicted HbA1c levels, thus impairing the robustness of this test relative to glycaemic control. This raises the possibility that alternative tools and new potential biomarkers of glycation would be better placed to assess the diagnosis and performance of diabetic treatments in patients treated with aspirin.

Transparency document

The Transparency document associated with this article can be found in the online version.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2015.04.003.

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